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Quil A fraction with low toxicity and use thereof

Field of invention

The present invention relates to the use of fraction A of Quil A together with at least one other adjuvant for the preparation of an adjuvant composition with synergistic immunomodulating activity.

Prior art

There is a great need for efficient adjuvant and vaccine delivery systems both for man and animal to be used for immune prophylactics or for immune therapy. For animal vaccines there are a number of different adjuvants including ISCOM and ISCOMATRIX adjuvanted vaccines. However, only aluminiumhydroxide and calciumphosphate adjuvants are commercially available in human vaccines, and an oil emulsion adjuvant (MF59) has recently been registered for a human influenza vaccine. Thus, there is a lack of efficient adjuvants, particularly for human vaccines. Adjuvants are not only important for enhancing the level immune response but even more for the quality or type of immune response, which has to match the type of infection the vaccine is intended to protect against. With regard to pathogens establishing themselves intracellularly like viruses, but also some bacteria and parasites, a so-called Th1 type of immune response is required for optimal immune protection, and in many cases a Th1 type of response is a prerequisite for immune protection. However, it is also now well established, that a pure Th1 or Th2 type of response may cause side effects, since a balance between the two types of the T helper cells are required for immune regulation. I.e. the Th1 response regulate the Th2 response e.g. by the production of IFN-γ and the Th1 response is regulated by the Th2 response e.g. by the production of the cytokine IL10. Thus, the Th1 - Th2 balance is essential to avoid side effects. To be able to induce correct type of immune response for protection against

the various pathogens a number of adjuvants will be required. A Th1 response is reflected by the IgG2a antibody response, and therefore used as a marker for Th1 t helper cell response. One important aspect for adjuvants is the safety including the fact that the immune response evoked shall have a quality to avoid side effects when a subsequent infection occurs after the vaccination. Severe side effects were the case with respiratory syncytial virus when an aluminium hydroxide adjuvanted formalin inactivated respiratory syncytial virus (RSV) vaccine was tried in children nearly 30 years ago. The vaccinated children became sicker and there was a higher death rate among them after natural infection with RSV than in non-vaccinated children.

Acute toxicity or side effects have been major concerns for both veterinary and particularly human use of quillaja saponins in vaccine preparations. Theses goals were only partially met with success, the purified fractions e.g., QA-21 (EP 0 362 279 B2) and combinations of fractions A and C (WO 96/11711, Iscotec-patent) were indeed chemically defined compared to "*Quillaja Saponaria* Molina" but they still caused some toxicity and side effects.

It has now turned out that fraction A of Quil A has a low toxicity, and in low dose enhance other adjuvants in suboptimal doses, which when used by themselves may be toxic in efficient doses. Thus, it facilitates the use of other adjuvants which, when used by themselves, might be toxic in doses they are efficient.

Summary of the invention

The present invention relates to the use of fraction A of Quil A together with at least one other adjuvant for the preparation of an adjuvant composition with synergistic effect to enhance the level of immune responses and immunomodulating activity. It especially concerns the use of fraction A of Quil A in a composition comprising iscom particles wherein the different fractions of Quil A are integrated into different iscom and iscom matrix particles.

Description of drawings

Fig 1-1

High dose (50μg) of QHC in matrix is toxic, while a high dose of QWT in ISCOM - MATRIX is non–toxic when supplemented to OVA to enhance the antibody response in Balb/C mice (see text). Both formulations enhance similar specific antibody responses against OVA as measured 3 weeks after the second immunisation by ELISA for the total IgG response (A) and in the IgG2a subclass (B)

Fig 1-2

Synergistic effects of QWT and QHC matrices when supplemented to OVA to enhance the antibody response in Balb/C mice (see text). The dose of the matrices QWT and C ranged as follows in group 1, no QWT or C; Gr. 2, 0.3μg QWT no C; Gr. 3, 0.3μg QWT + 2μg C; Gr. 4, 10μg QWT no C; Gr. 5, 10μg QWT 2μg C. The dose of OVA was 10μg. There were 8 mice per group, which were immunised twice 4 weeks apart s.c. with respective formulation. The antibody titres were measured by ELISA against OVA:

A Total IgG 3 weeks after the first immunisation
B IgG2a 2 weeks after the second immunisation
C IgG1 2 weeks after the second immunisation

Fig 2-1

Toxicity of QWT and AC (i.e.703) respiratory syncytial virus (RSV) ISCOMs measured by survival rate in newborns (1 week old) mice after one intraperitoneal injection with 1μg ISCOM (protein). The protein/saponin ratio is 1/1.

Fig 3-1

Antibody response of newborn (1 week old) and adult mice after one intraperitoneal immunisation and a subsequent boost after 3 weeks with 1μg ISCOM (protein). The protein/saponin ratio is 1/1.

Fig 4 – 1

Cytotoxic T cell (CTL) response after one intraperitoneal immunisation with 1µg ISCOM (protein). The protein/saponin ratio is 1/1. The spleen cells were collected 1 and 3 weeks after the intraperitoneal immunisation.

Fig 5-1

QWT matrix is less toxic on VERO cells (a monkey cell line) than 703 matrix and C matrix after exposure for 72 hrs in culture measured by growth rate proportional (%) to non-exposed cell cultures. QWT matrix is well tolerated at all concentrations tested i.e. up to 1300µg. No cell growth is recorded in cell cultures exposed 800µg of 703 matrix or 45µg of QHC matrix.

A. Exposure of VERO cells to QWT matrix and 703 matrix as indicated.

B. Exposure of VERO cells to QHC matrix as indicated.

Fig 5-2

QWT matrix is less toxic on spleen cells obtained from mice than C matrix after exposure for 72 hrs in culture measured by growth rate measured by a colorimetric method as described in the text. The growth rate is compared with spleen cells grown in medium alone or together with mitogen Con A.

A. Exposure of spleen cells to QWT matrix in decreasing doses from 10 to 1,25µg as indicated.

B. Exposure of spleen cells to QHC matrix in decreasing doses from 10 to 1,25µg as indicated.

Figure 6

This figure shows the preparation of fractions A, B and C by HPLC;

Detailed description of the invention

The invention relates to the use of fraction A of Quil A together with at least one other adjuvant for the preparation of an adjuvant composition with synergistic effect to enhance the level of immune responses immunomodulating activity. It especially relates to the use of fraction A of Quil A together with one or more other adjuvants for (alternative and in low dose enhance the effect adjuvants in suboptimal dose, which may be toxic in efficient doses, which facilitates its use), The effect is that it is lowering the toxicity of these other adjuvants in useful doses. Thus, the other adjuvants are preferably those which have a substantial toxicity which could be lowered.

The at least one other adjuvant may be chosen preferably from saponins, naturally occurring, synthetic or semisynthetic saponin molecules derived from crude saponin extract of Quillaja saponaria Molina; e.g. saponins and saponin fractions from Quil A, cell wall skeleton, blockpolymers, e.g. hydrophilic block copolymers, e.g. CRL-1005, TDM (Threhalose di mucolate), lipopeptides, LPS and LPS-derivatives, Lipid A from different bacterial species and derivatives thereof, e.g., monophosphoryl lipid A. CpG variants, CpGODN variants, endogenous human animal immunomodulators, e.g. GM-CSF. IL-2, adjuvant active bacterial toxins, native or modified, e.g. cholera toxin CT, LT, CTA1 or Bordetella pertussis toxin, BP.

The saponin fractions from Quil A other than fraction A may be the B and C fractions described in WO 96/11711, the B3, B4 and B4b fractions described in EP 0 436 620 The fractions QA1-22 described in EP 0 3632 279 B2, Q-VAC (Nor-Feed, AS Denmark), *Quillaja Saponaria* Molina Spikoside (Isconova AB, Ultunaallén 2B, 756 51 Uppsala, Sweden)

The fractions QA-1-2-3-4-5-6-7-8-9-10-11-12-13-14-15-16-17-18-19-20-21 and 22 of EP 0 3632 279 B2, Especially QA-7, 17-18 and 21 may be used. They are obtained as

described in EP 0 3632 279 B2, especially at page 6 and in Example 1 on page 8 and 9.

Fractions A, B and C described in WO 96/11711 are prepared from the lipophilic fraction obtained on chromatographic separation of the crude aqueous *Quillaja Saponaria* Molina extract and elution with 70% acetonitrile in water to recover the lipophilic fraction. This lipophilic fraction is then separated by semipreparative HPLC with elution using a gradient of from 25% to 60% acetonitrile in acidic water. The fraction referred to herein as "Fraction A" or "QH-A" is, or corresponds to, the fraction, which is eluted at approximately 39% acetonitrile. The fraction referred to herein as "Fraction B" or "QH-B" is, or corresponds to, the fraction, which is eluted at approximately 47% acetonitrile. The fraction referred to herein as "Fraction C" or "QH-C" is, or corresponds to, the fraction, which is eluated at approximately 49% acetonitrile.

Preferably the at least other adjuvant is subfragment C from Quil A.

The adjuvant fraction A of Quil A may be integrated into one iscom particle and the at least one other adjuvant is preferably also integrated into another different iscom particle. Thus the adjuvants may be integrated into each a different iscom particle and then mixed in a composition. The iscom particle may be an iscom complex or an iscom matrix complex made from any saponin.

The adjuvant fraction A and the other at least one adjuvant may however also be coupled on to the different iscom particles or one of the adjuvants may be mixed with the iscom particles.

The adjuvant fraction A and the other at least one adjuvant may also be formulated in liposomes or with oil-based adjuvant formulation or with a non-ionic block polymer presented in another particulate formulations such as PLG, starch, or in free form.

Iscom contains at least one glycoside, at least one lipid and at least one type of antigen substance. The lipid is at least a sterol such as cholesterol and optionally also phosphatidyl choline. This complexes may also contain one or more other immunomodulatory (adjuvant-active) substances, and may be produced as described in EP 0 109 942 B1, EP 0 242 380 B1 and EP 0 180 564 B1.

An iscom matrix, comprises at least one glycoside and at least one lipid. The lipid is at least a sterol such as cholesterol and optionally also phosphatidyl choline. The iscom complexes may also contain one or more other immunomodulatory (adjuvant-active) substances, not necessarily a saponin, and may be produced as described in EP 0 436 620 B1.

In a preferred formulation iscoms and iscom matrix have been formulated with fraction A and C of Quillaja in different iscom particles, which cause minimal side effects (see the examples). These iscoms have been compared with a formulation comprising 70% of fraction A and 30% of fraction C of Quil A called 703 and produced according to WO 96/11711, which is in clinical trail in man for a human influenza virus vaccine. According to WO 96/11711 the A and C fractions are integrated into the same particle. The toxicity study was carried out in newborn mice, which are much more sensitive than adult mice. The study shows that the newborn mice better tolerate the new iscoms produced from fraction A of Quil A than the 703 formulation. Furthermore, the efficacy of the new formulations according to the invention is tested with antigens from a pathogen i.e. hRSV and with a weak antigen i.e. ovalbumin (OVA). A synergistic effect of fraction A of Quil A in a matrix formulation named QWT is shown in example 1.

A composition according to the invention may comprise the adjuvant fraction A from Quil A and the at least one other adjuvant in any weight ratios. Preferably fraction A of Quil A is from 2-99weight %, preferably 5-90 weight% and especially 50-90 weight% counted on the total amount of adjuvants.

One preferred iscom composition comprises 50-99,9% of fragment A of Quil A and 0,1-50% of fragment C of Quil A counted on the total weight of fractions A and C. Especially the composition comprises 75-99,9% of fragment A of Quil A and 0,1-25% of fragment C of Quil A counted on the total weight of fractions A and C. Most preferred composition comprises 91-99,1% of fragment A of Quil A and 0,1-9% of fragment C of Quil A counted on the total weight of fractions A and C, especially 98,0-99,9 % of fraction A 0,1-2,0% of fraction C.

The composition may further comprises a pharmaceutically acceptable carrier, diluent, excipient or additive.

For the purposes of identification of Fractions A, B and C referred to herein, reference may be made to the purification procedure of Example 1. In general terms, in this procedure Fractions A, B and C are prepared from the lipophilic fraction obtained on chromatographic separation of the crude aqueous Quil A extract and elution with 70% acetonitrile in water to recover the lipophilic fraction. This lipophilic fraction is then separated by semipreparative HPLC with elution using a gradient of from 25% to 60% acetonitrile in acidic water. The fraction referred to herein as "Fraction A" or "QH-A" is, or corresponds to the fraction, which is eluted at approximately 39% acetonitrile. The fraction referred to herein as "Fraction B" or "QH-B" is, or corresponds to, the fraction, which is eluted at approximately 47% acetonitrile. The fraction referred to herein as "Fraction C" or "QH-C" is, or corresponds to, the fraction, which is eluted at approximately 49% acetonitrile.

When prepared as described herein, Fractions A, B and C of Quil A each represent groups or families of chemically closely related molecules with definable properties. The chromatographic conditions under which they are obtained are such that the batch-to-batch reproducibility in terms of elution profile and biological activity is highly consistent.

All publications mentioned herein are incorporated by reference. By the expression "comprising" we understand including but not limited to. The invention will now be described by the following non-limiting examples. The scope of the invention is rather what the skilled person would interpret from the disclosure and found equivalent or a natural development thereof.

Example 1

In this experiment it is emphasised that that QWT is well tolerated and has a strong immune enhancing and immune modulatory capacity. Ovalbumin (OVA) is used because it is a weak antigen and as such it does not induce a Th1 type of response. QWT is compared with QHC, since it is evaluated in human clinical trials.

Materials and methods

Formulation of QHC, QWT and 703-matrix ISCOMs

A mixture of phosphatidyl choline (PC) and cholesterol (C)(15 mg/ml of each) is prepared in 20% MEGA-10 in water. The preparation is heated to 60°C and treated with light sonication until all lipid is solubilised.

Quillaja saponin is dissolved to 100 mg/ml in water. The 703 mixture contains 7 parts (by weight) of Fraction A and 3 parts of Fraction C.

QWT saponin contains Fraction A alone.

703-matrix. 5 ml of PBS is mixed with 10 mg of the PC/C mixture (667 microliters), 35 mg 703 (350 microliters) is added, the mixture is mixed and PBS is added to a final total volume of 10 ml. The mixture is extensively dialysed against PBS using a Slide-A-Lyser (3-15 ml, Pierce) dialysis casette.

QWT-matrix. and QHC-matrix 5 ml of PBS is mixed with 10 mg of each PC and C (667 microliters), 40 mg QWT (400 microliters) and 30 mg of QHC (300 microliters) respectively is added, the mixture is mixed and PBS is added to a final total volume of 10 ml. The mixture is extensively dialysed against PBS using a Slide-A-Lyser (3-15 ml, Pierce) dialysis casette.

Experimental design

Group 1 consisted of 8 mice immunised twice 4 weeks apart subcutaneously (s.c.) with 10μg OVA ajuvanted with 50 μg QWT matrix. Group 2 had the same number of mice immunised by the same procedure but the adjuvant was 50 μg QHC matrix.
Sera were collected before first immunisation and 3 weeks after and 2 weeks after the boost.

Antibody determination

The specific OVA serum antibody responses were determined by ELISA both for total IgG response and in the IgG2a subclasses as described before (Johansson, M and Lövgren-Bengtsson (1999) Iscoms with different quillaja saponin components differ in their immunomodulating activities. Vaccine 19, 2894-2900).

Results

All mice immunised with OVA adjuvanted with QWT matrix survived and did not develop any sign of discomfort. Out of 8 mice immunised with OVA adjuvanted with QHC matrix 4 mice (50%) died.

There is no significant difference between the groups with regard total antibody responses (Fig 1-1A), but there is more spread of the antibody titres between the animals in group 1, i.e. mice immunised with OVA adjuvanted with QWT.

There was no difference in mean titres in the IgG2a subclass between group 1 and 2 (Fig 1-1B).), but there is more spread of the antibody titres between the animals in group 2, i.e. mice immunised with OVA adjuvanted with QHC.

In the second experiment of this example it was explored whether QWT matrix can benefit from the complementation of another adjuvant, or it facilitate the use of a more toxic adjuvant. The IgG2a response reflects that the Th2 type of lymphocytes are involved. The dose of QWT and C matrices ranged as follows; in group 1, no QWT or QHC; Gr. 2, 0.3μg QWT no QHC; Gr. 3, 0.3μg QWT + 2μg QHC; Gr. 4, 10μg QWT no QHC; Gr. 5, 10μg QWT +2μg QHC. The dose of OVA was 10μg. There were 8 mice per group, which were immunised twice 4 weeks apart s.c. with respective formulation. (example 8 Fig 2 A, B and C).

Sera were collected 3 weeks after the first immunisation and 2 weeks after the boost.

The specific OVA serum antibody responses were determined by ELISA for total IgG response and in the IgG2a and IgG1 subclasses as described (Johansson, M and Lövgren-Bengtsson (1999). Iscoms with different quillaja saponin components differ in their immunomodulating activities. Vaccine 19, 2894-2900).

Results

After the first immunisation no antibody response was recorded in mice receiving non-adjuvanted OVA or OVA adjuvanted with 0.3μg of QWT matrix with and without 2μg of QHC matrix (Fig 1-2A)

After the second immunisation a low response was detected in 3 out of 8 mice immunised with non-adjuvanted OVA in the IgG1 subclass(Fig 1-2B), but no response was recorded in the IgG2a subclass. Neither was antibody responses recorded in the IgG2a subclass with the lowest adjuvant doses of QWT matrix i.e. 0.3μg with and without 2μg of QHC matrix (Fig 1-2B). There was a clear enhancement of the

antibody response in the IgG2a subclass, when the low dose of 2μg QHC matrix was added to the 10 μg of QWT matrix (Fig 1-2B).

Conclusion

QWT has a low toxicity and still a strong modulatory effect, when included in ISCOM-matrix as shown by promoting a strong TH1 type of response, in contrast to the non-adjuvanted or the very low adjuvanted OVA, which only elicited antibody response in the IgG1 subclass. It is also shown that the QWT matrix synergies with a low dose of QHC matrix. This fact is important, because QWT makes it possible to optimise the adjuvant effect and minimise the side effects of other adjuvants.

Example 2

Respiratory syncytial virus (RSV) is a major pathogen for young children (hRSV) but also for elderly. A closely related virus (bRSV) is a pathogen for young calves causing sever disease and high economical losses for calf breeders. The envelope proteins of hRSV were selected as model antigens, because they represent antigens from a pathogen for which a vaccine is lacking and for which there is a great need. The newborn mouse represents a model for the newborn, and a very sensitive animal, which requires a vaccine formulation virtually free of side effects, and a model in which important immunological reactions can be measured because of available reagents techniques. An early vaccine against hRSV was tested in children, but it did not protect against disease. On the contrary it exacerbated disease when a subsequent natural infection occurred. In this experiment we have selected 703 as a quillaja component in the ISCOM to compare with the present invention, because a 703 vaccine formulation is in human trials, thus a candidate for human vaccines. In the present experiment the toxicity of QWT ISCOMs and 703 ISCOMs is compared.

Materials and methods

Formulation of 703 and QWT RSV-ISCOMs

RSV ISCOMs with different *Quillaja* saponin compositions (A, C and AC i.e., ISCOPREPTM703) were prepared from sucrose gradient purified HRSV, essentially using the method described previously [17,18]. Briefly, 2 ml (1.6 mg/ml) purified RSV was solubilized with OG (1-O-n-Octyl-ß-D-glucopyranosid, C14H28O6, Boehringer, Mannheim, GmbH, FRG) at a final concentration of 2% (w/v) for 1 h at 37 °C under constant agitation. The solubilized virus was applied onto a discontinuous sucrose gradient of 2 ml 20% sucrose layer containing 0.5% OG, over a cushion of 50% sucrose. After centrifugation at 210,000 g at 4 °C in a Kontron TST-41 rotor for 1 h, the sample volume together with the 20% sucrose layer containing viral proteins were collected, and extra lipids i. e. cholesterol and phosphatidylcholine, and *Quillaja* saponin, i.e. QH-A or QH-C or ISCOPREPTM703 was added in proportions of protein: cholesterol: phosphatidylcholine : *Quillaja* saponin = 1:1:1:5 calculated by weight . After extensive dialysis against 0.15 M ammonium acetate at 4 °C for 72 h, the ISCOMs were purified by centrifugation through 10% sucrose at 210,000 g in Kontron TST-41 rotor at 10 °C for 18 h. The pellet containing the ISCOMs was re-suspended in 200 µl PBS. Protein concentration was determined by amino acid analysis (Aminosyraanalyslaboratoriet, Uppsala, Sweden). Samples were submitted for negative staining electron microscopy. No morphological differences were observed among the three ISCOMs. All showed typical ISCOM structures, i.e. cage-like spherical particles with a diameter of around 40 nm. The RSV antigens and ISCOM structures were found in the same fraction of a sucrose gradient after centrifugation.

Experimental design

One litter of at least 7 newborn (one week old) mice per group were injected intraperitoneally (i.p.) once with either a formulation 703 ISCOMs or QWT ISCOMs The dose groups of each quillaja component ranged between 0.11µg and 1µg

measured as protein content (Fig 2-1). The ratio QWT or 703 (quillaja saponin) protein *is* 1/1. The pups were observed for 15 days after i.p. injection. It should be noted that the i.p. injection is a rough mode of administration and mice are much more sensitive for i.p. injection than for intramuscular and subcutaneous modes of administrations.

Results

Doses of 0.66 and 1μg killed 65 resp. 50% of the mice injected with the 703 ISCOMs, while all the mice injected with the QWT ISCOMs survived including those receiving 1μg of QWT ISCOMs.

Conclusion

The QWT ISCOM is well tolerated even by a harsh route as the i.p. route in a very sensitive animal model. It is better tolerated than a formulation being in human trails.

Example 3

In this example the serum antibody response was tested with the envelope proteins G and F of hRSV as a model for vaccine antigen. The hRSV antigens were selected because hRSV represents antigens from a pathogen for which a vaccine is lacking and for which there is a great need. The newborn mouse represents a model for the newborns, which are immunologically immature requiring an adjuvant system with potent immune modulatory capacity (WO97/30727). Furthermore, a newborn mouse represents an animal system, which is very sensitive and requires a vaccine formulation virtually free of side effects. Similar vaccine formulations were tested as described in example 2 i.e. the QWT AND 703 ISCOMs.

Materials and methods

Formulation of QWT and 703 RSV-ISCOMs

See example 2

Experimental design

One-week-old mice and adult mice (BALB/C) were distributed into 2 groups of newborns and 2 groups of adults. One litter of newborns with minimum of 7 animals per group and 8 adults were immunised i.p. with 1μg of hRSV in the QWT ISCOMs or in the 703 ISCOM formulation. One group of newborns and 1 group of adult mice were immunised once, while 1 group of newborns and 1 group of adult mice were boosted 3 weeks after the first immunisation with the same formulations by the same mode. All experiments were repeated once.

Sera were collected before boost and week 7 of life i.e. 3 weeks after boost. Because of the small size of the newborns the sera were pooled from one group.

Antibody determination

The specific RSV serum antibody responses were determined by ELISA in both IgG1 and IgG2a subclasses as described using 0.1ul of formalin killed RSV virus as coating antigen (Johansson, M and Lövgren-Bengtsson (1999) Iscoms with different quillaja saponin components differ in their immunomodulating activities. Vaccine 19, 2894-2900).

Results

The results are illustrated in Fig 3-1. After one immunisation both adults and newborns responded with RSV specific IgG1 antibodies measured by ELISA. After one immunisation the QWT ISCOMs induced higher RSV specific IgG1 antibody response in the newborn than the 703 ISCOM. Otherwise, there were no clear differences between the two ISCOM formulations as regards to their capacity to induce IgG1 and IgG2a RSV specific antibody responses in adults or in newborns. The antibody titres in general were 10-fold higher in the adults than in newborns. The IgG2a response to RSV was insignificant after one immunisation in newborns regardless they were

immunised with QWT or 703 ISCOMs. RSV specific IgG2a were clearly detected after one immunisation in adults.

Conclusion

The serum antibody responses were at least as high after 1 as well as after 2 immunisations of newborns or adults with the QWT ISCOM formulation as after the same immunisation schedules with the 703 ISCOM. In view of the results of example 2, showing that the QWT ISCOM has a considerably lower toxicity than the 703 ISCOM, the QWT ISCOM is preferred for vaccine formulation.

Example 4

Cytotoxic T lymphocytes (CTL) are essential for the immune defence against intracellular pathogens. Above all virus-infected cells are targets for CTL by killing the infected cells. Consequently, CTL is an important arm of the immune defence against viral infections. This example shows that QWT ISCOMs containing hRSV envelope antigens specifically induce and efficiently prime for memory CTL both in newborn and adult mice. It is surprising, that the QWT ISCOMs induced CTL memory as efficiently in the newborns as in adults in view of their immature immune system.

Materials and methods

Formulation of QWT and 703 RSV ISCOMs

The QWT and 703 ISCOMs were prepared as described in example 2.

Animals and experimental design

One litter of newborns with at least 7 animals were used for each experiment. (8 adult BALB/C (H-2Kd) mice. Each experiment was carried out twice. One-week-old mice or adult mice were injected i.p. with 1μg of QWT ISCOMs. One week resp. 3 weeks after immunisation spleen cells (effector cells) were cultured (restimulated) for 6 days in vitro with HRSV infected (BCH4)-fibroblast (target cells). The ratio of

effector/target (E/T) ranged from 2 to 100 (Fig 4-1). The target cell lysis was measured by Cr51 release and expressed as % specific lysis (%SL) according to standard procedure. 100% lysis was measured as Cr51 release from detergent treated cells. The background was the lysis caused by uninfected fibroblasts (BC) (see Fig4-1).

Results

Already 1 week after priming of newborn and adult mice with QWT ISCOMs their splenocytes generated to restimulation in vitro with hRSV infected fibroblasts (BCH4) strong cytotoxic T cell response (Fig 4-1). No lysis was observed against uninfected target cells (BC in Fig 4-1).

Conclusion

RSV- QWT ISCOMs induce strong cytotoxic T cell responses in 1-week-old mice and in adult mice. Strong specific cytotoxicity is observed already 1 week after one immunisation. In view of the strong adjuvant effect of QWT ISCOMs and its low toxicity, this vaccine delivery and adjuvant system is very likely to be valuable for both human and animal vaccines.

Example 5

Quillaja saponins have been shown to have strong adjuvant effects, but they have caused side effects by their lytic properties, which can be measured by lysis of red blood cells. Toxic effects of any kind prevent the cell growth or proliferation of living cells. It is well established that QHC and less purified quillaja saponins like Quil A lyses red blood cells (Rönnberg B, Fekadu M and Morein B, Adjuvant activity of non-toxic Quillaja saponaria Molina components for use in ISCOM matrix, Vaccine, 1995 13, (14): 1375-82.). It is also clear that lytic effect of quillaja saponins causes local reactions when injected. One way to avoid lytic effects of saponins is to include them into ISCOM matrix. Furthermore, the side effects can be reduced by selection of

quillaja saponin, which causes comparatively low side effect. In this example the effect of QWT matrix is tested on VERO cells, which is a primate cell line, and it is compared with QHC and 703 matrix formulations. In a second experiment spleen cells from mice were exposed to QWT and QHC matrices. The spleen cells are representative for the lymphatic system essential for the induction of immune responses. The alamarBlue Assay is used, which measures quantitatively the proliferation of the cells based on detection of metabolic activity.

Material and methods

Cells and cell growth. Vero cells were cultured in RPMI 1640 medium (National Veterinary Institute Uppsala Sweden) supplemented with 7% fetal calf serum (obtained as above). After outgrowth on 75 cm^2 flasks (Corning-Costar, Acton MA, USA) the cells are detached from the plastic surface and diluted to 25 to 30 000 per ml, and distributed in 100 µl portions per well in 96 well cell culture plates (Nunc A/S, Roskilde, Denmark). The cultures are incubated in CO^2 atmosphere for 24, 48 and 72 hours. Matrix prepared with QWT, or 703 or QHC were diluted in medium from 0 to 1300 µg per ml. The cell cultures were emptied from medium and the matrix dilutions were added to the wells. As control only medium was used. The test was carried out with the formulations to be tested for incubation periods of 24, 48 and 72 hours. Most suitable time period was 72 hours, which is presented here. The controls are considered as 100% growth.

Recording of cell growth. The AlamarBlue assay (Serotec Ltd, Oxford UK), wich measures quantitatively the proliferation of the cells based o detection of metabolic activity was used according to the description of the manufacturer.

Results

After 72 hours incubation of the cell cultures with QWT matrix at a concentration 1300 µg per ml a cell growth of 80% was recorded compared to the control cultures,

while the cell growth had declined to 0% when exposed to 703 matrix at a concentration of 800 µg per ml. The cell growth had declined to 0% when exposed to QHC matrix at concentration of 40 µg per ml. Fig 5-1illustrates one experiment out of 3 with similar results.

Conclusion

QWT matrix is well tolerated by the cells and has very low cell toxic effect.

In a second experiment spleen cells were exposed to QWT and QHC matrices.

Material and methods

Cells and cell growth. Spleen cells from BalbC mice were cultured in RPMI 1640 medium (National Veterinary Institute, Uppsala, Sweden) supplemented with 7% fetal calf serum in 96-well cell culture plates (Nunc, Roskilde Denmark). The test was carried out on the spleen cells with the formulations QWT and QHC for incubation periods of 24, 48 and 72 hours. Most suitable period was 72 hours, which is presented here. The controls are considered as 100% growth.

Recording of cell growth. The alamarBlue Assay is used, which measures quantitatively the proliferation of the cells based on detection of metabolic activity was used according to the description of the manufacture.

Results

After 72 hours exposure of the spleen cell cultures to QWT matrix at a concentration 10 µg per ml a cell growth of 80% was recorded compared to the non-exposed spleen cell (control) cultures, while the cell growth had declined close to 0% when exposed to QVC matrix at a concentration of 2 µg per ml (Fig5-2 A and B). Fig 5-2 1illustrates one experiment out of 3 with similar results.

Example 5 Preparation of *Quillaja Saponaria* Molina subfragment saponins.

Purification of crude *Quillaja Saponaria* Molina extract to fractions A, B and C.

A solution (0.5ml) of crude Quillaja bark extract in water (0.5 g/ml) is pre-treated on a sep-pak column (Waters Associates, MA).

The pre-treatment involves washing of the loaded sep-pak column with 10% acetonitrile in acidic water in order to remove hydrophilic substances. Lipophilic substances including QH-A, QH-B and QH-C are then eluted by 70% acetonitrile in water.

The lipophilic fraction from the sep-pak column is then separated by a semipreparative HPLC column (CT-sil, C8, 10 X 250mm, ChromTech, Sweden).

The sample is eluted through the column by a gradient from 25% to 60% acetonitrile in acidic water. Three fractions are collected from the HPLC column during the separation. The residues after evaporation of these three fractions constitute QH-A, QH-B and QH-C.

The fractions designated QH-A, QH-B and QH-C were eluted at approximately 39, 47 and 49% acetonitrile respectively. The exact elution profile and conditions are shown in Figure 6.

Patent claims

1. Use of fraction A of Quil A together with at least one other adjuvant for the preparation of an adjuvant composition with synergistic immunomodulating activity.

2. Use according to claim 1 wherein the at least one other adjuvant is chosen from saponins, naturally occurring, synthetic or semisynthetic saponin molecules derived from crude saponin extract of Quillaja saponaria Molina; e.g. saponins and saponin fractions from Quil A, cell wall skeleton, blockpolymers, e.g. hydrophilic block copolymers, e.g. CRL-1005, TDM (Threhalose di mucolate), lipopeptides, LPS and LPS-derivatives, Lipid A from different bacterial species and derivatives thereof, e.g., monophosphoryl lipid A. CpG variants, CpGODN variants, endogenous human animal immunomodulators, e.g. GM-CSF. IL-2, adjuvant active bacterial toxins, native or modified, e.g. cholera toxin CT, LT, CTA1 or Bordetella pertussis toxin, BP.

3. Use according to claim 2 wherein the subfragments from Quil A are chosen from fraction C or B of Quil A.

4. Use according to any of claims 1 –3, wherein the fraction A of Quil A is integrated into one iscom particle and the at least one other adjuvant is integrated into another iscom particle.

5. Use according to claim 4, wherein the iscom particle is an iscom complex.

6. Use according to claim 4, wherein the iscom particle is an iscom matrix complex.

7. Use according to any of claims 4-6, wherein the composition comprises 50-99,9% of fragment A of Quil A and 0,1-50% of fragment C of Quil A counted on the total weight of fractions A and C.

8. Use according to any of claim 7, wherein the composition comprises 75-99,9% of fragment A of Quil A and 0,1-25% of fragment C of Quil A counted on the total weight of fractions A and C.

9. Use according to claim 5, wherein the iscom particle comprises 91-99,1% of fragment A of Quil A and 0,1-9% of fragment C of Quil A counted on the total weight of fractions A and C.

10. Use according to any of claims 1-9, wherein the composition further comprises a pharmaceutically acceptable carrier, diluent, excipient or additive.

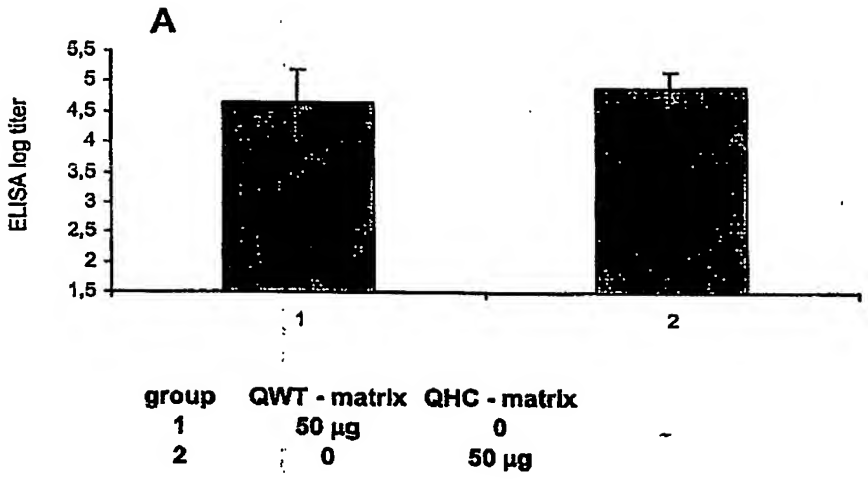
ABSTRACT

The invention relates to the use of fraction A of Quil A together with at least one other adjuvant for the preparation of an adjuvant composition with synergistic immunomodulating activity.

The at least one other adjuvant may be chosen from saponins, naturally occurring, synthethic or semisynthetic saponin molecules; e.g. saponins and saponin fractions from Quil A, cell wall skeleton, blockpolymers, TDM, lipopeptides, LPS and LPS-derivatives, Lipid A from different bacterial species and derivatived thereof, e.g., monophosphoryl lipid A. CpG variants.

Fig 1.

Fig 1-1



group	QWT - matrix	QHC - matrix
1	50 µg	0
2	0	50 µg

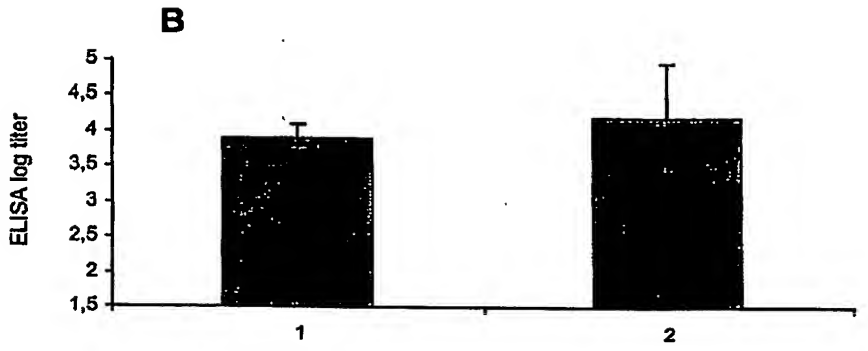


Fig 1-2

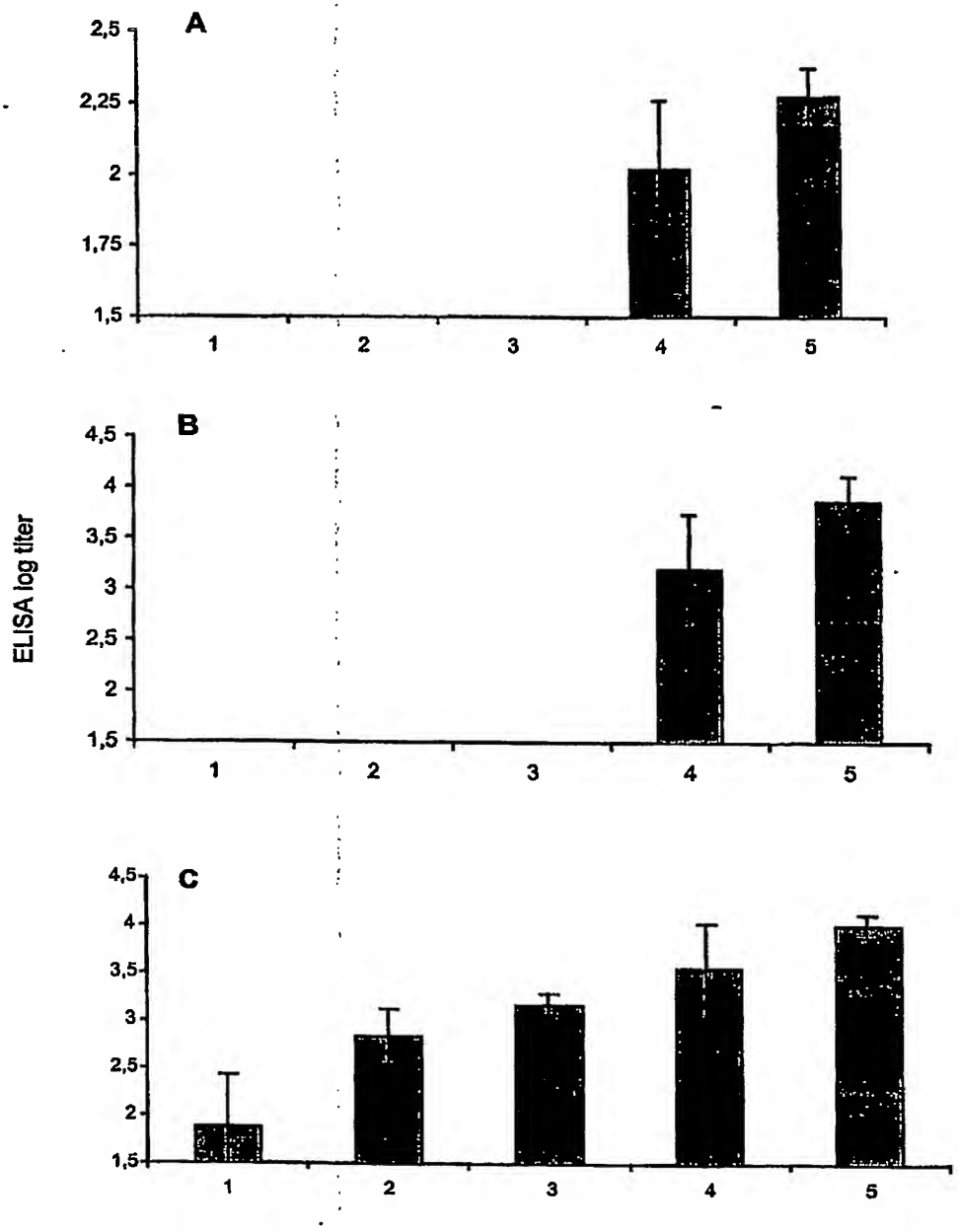
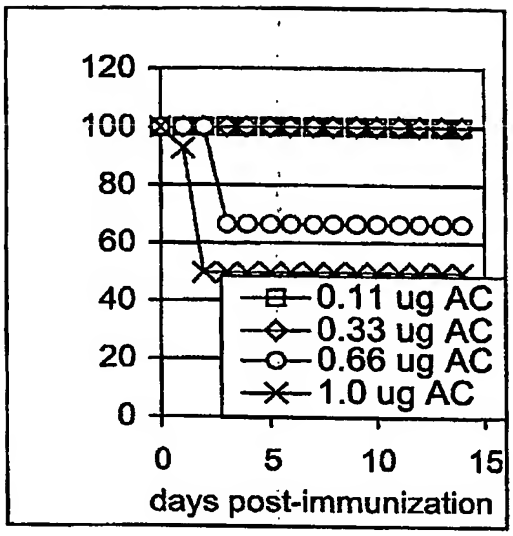


Fig 2-1


120
100
80
60
40
20
0
0
5
10
15
days post-immunization
0.11 ug AC
0.33 ug AC
0.66 ug AC
1.0 ug AC

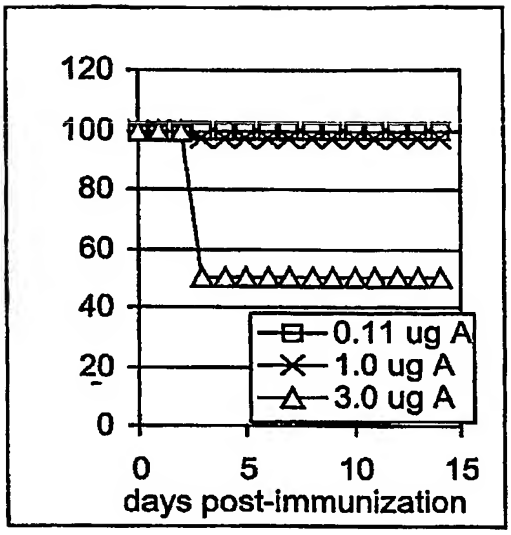
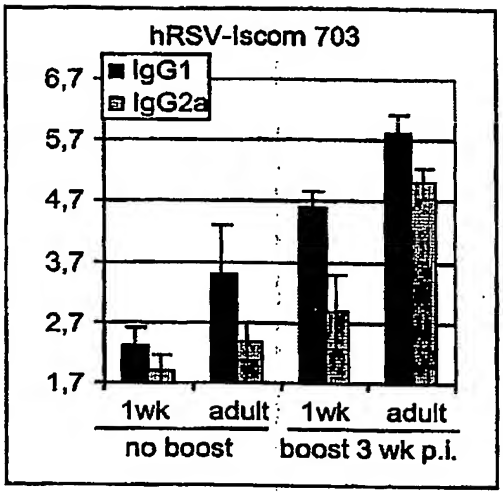

120
100
80
60
40
20
0
0
5
10
15
days post-immunization
0.11 ug A
1.0 ug A
3.0 ug A

Fig 3-1


hRSV-Iscom 703
IgG1
IgG2a
6,7
5,7
4,7
3,7
2,7
1,7
1wk
adult
1wk
adult
no boost
boost 3 wk p.i.

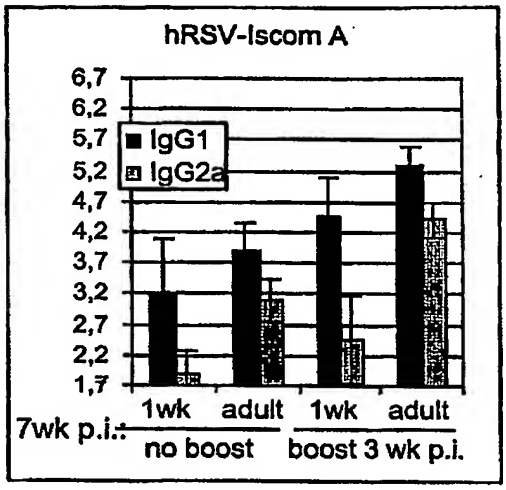
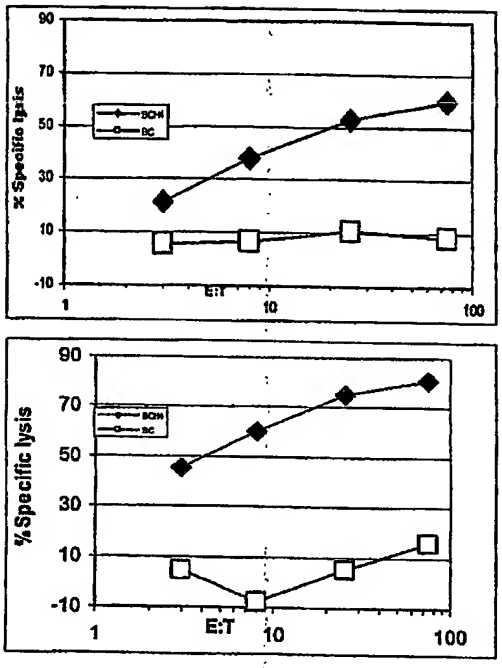
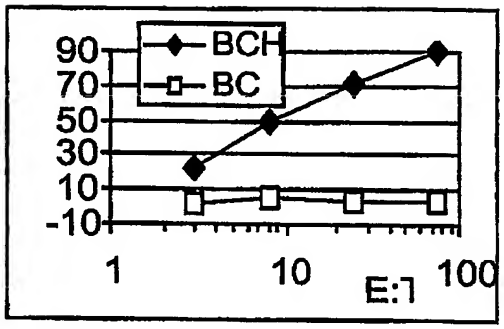

hRSV-Iscom A
6,7
6,2
5,7
5,2
4,7
4,2
3,7
3,2
2,7
2,2
1,7
IgG1
IgG2a
7wk p.i.:
1wk
adult
1wk
adult
no boost
boost 3 wk p.i.

Fig 4-1


% Specific lysis
BCH
BC
90
70
50
30
10
-10
1
E:T
10
100


BCH
BC
90
70
50
30
10
-10
1
10
E:T
100

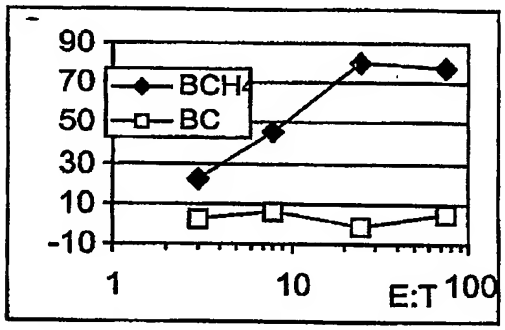

BCH
BC
90
70
50
30
10
-10
1
10
E:T
100

Fig 5-1

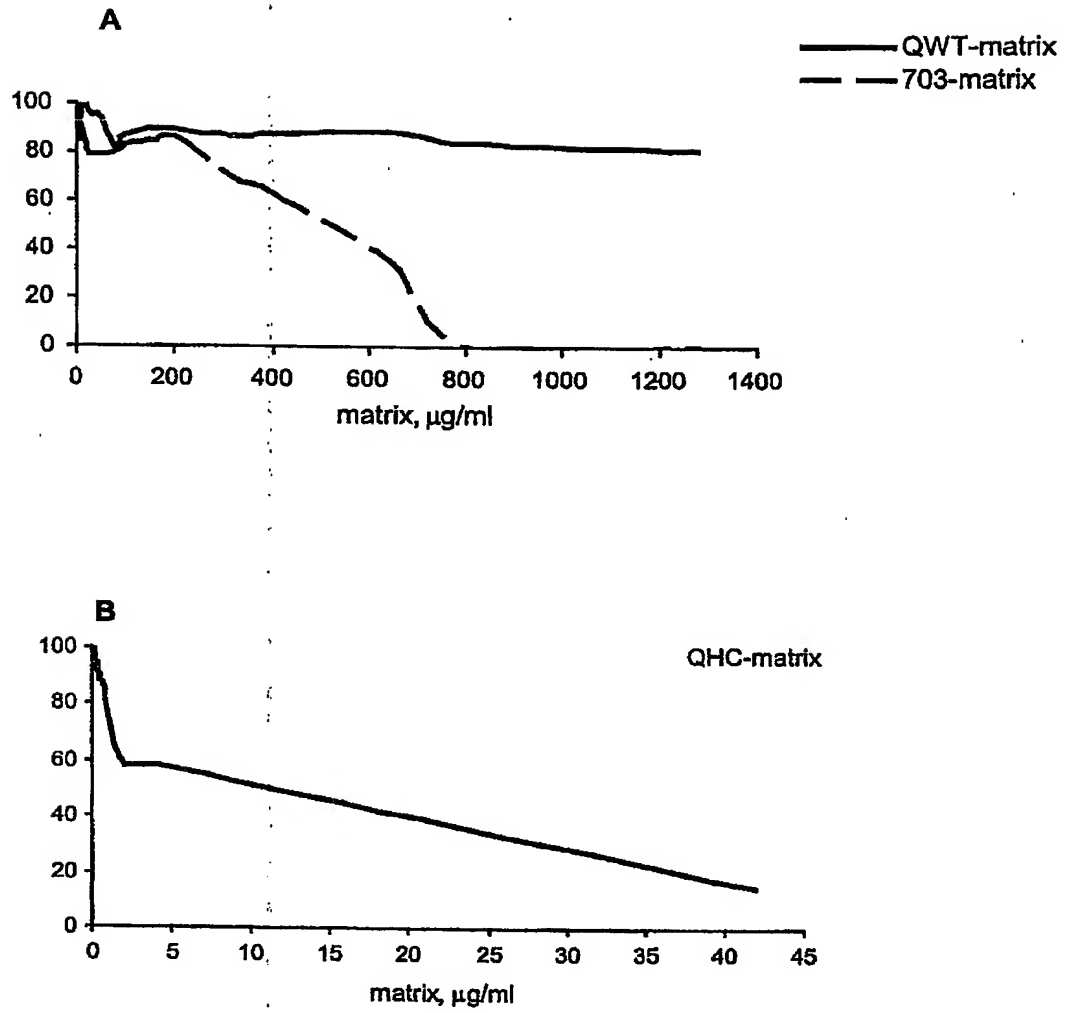

A
QWT-matrix
703-matrix
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
matrix, µg/ml
B
QHC-matrix
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
matrix, µg/ml

Fig 5-2

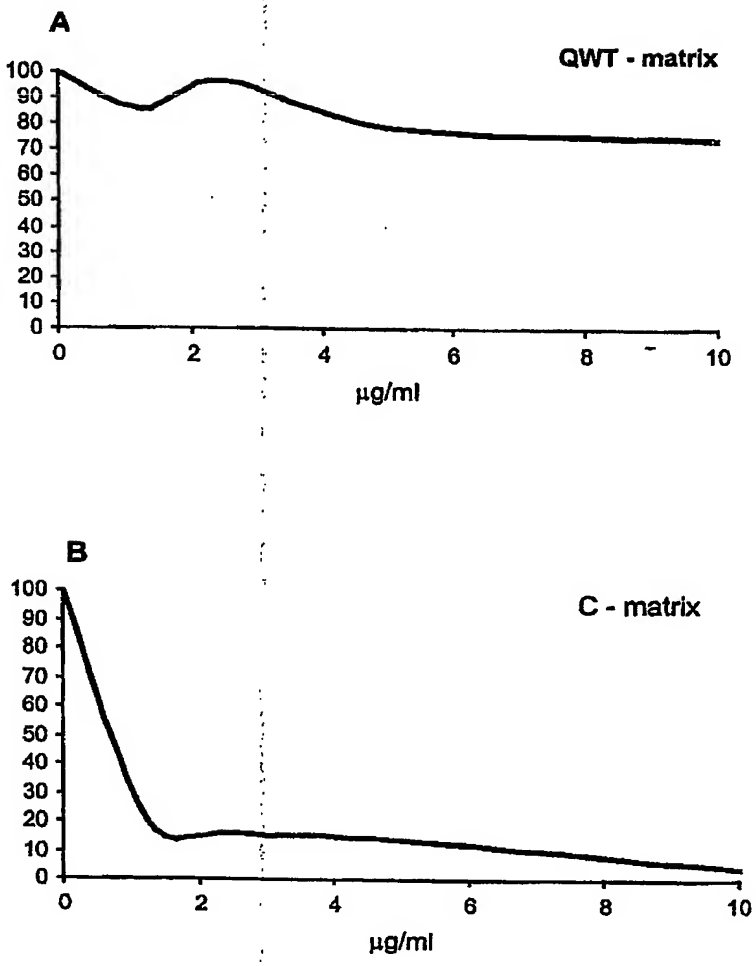
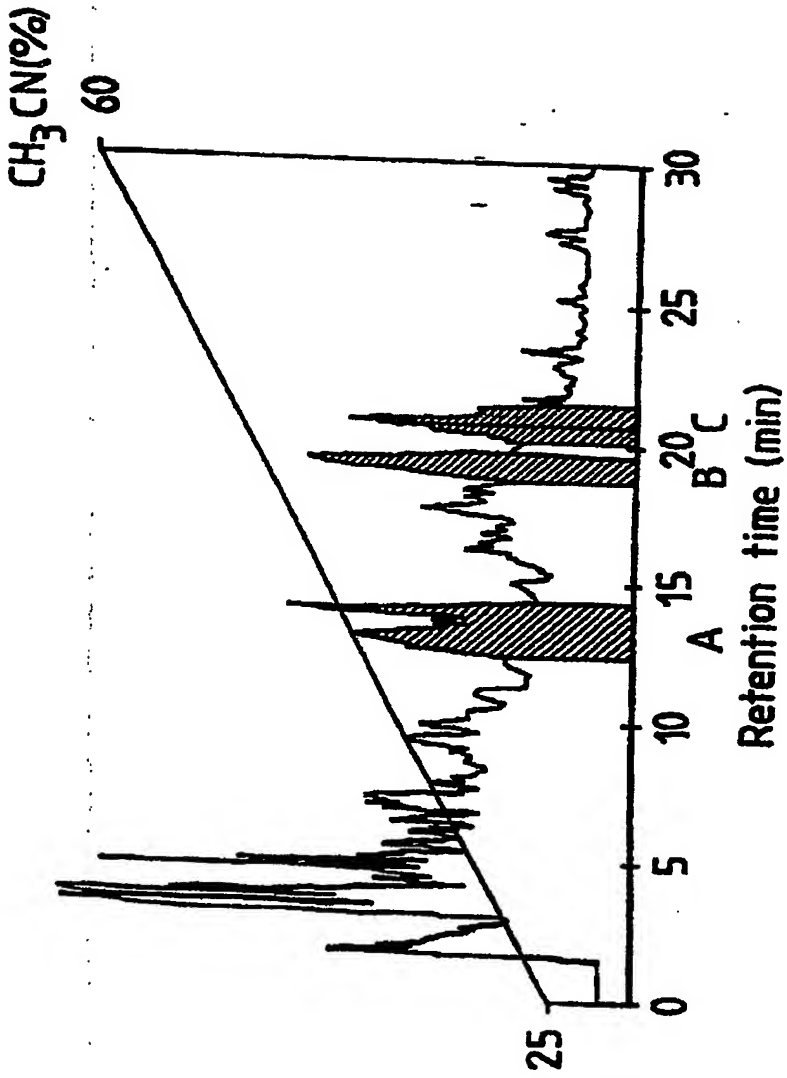

A
QWT - matrix
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
µg/ml
B
C - matrix
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
µg/ml

Fig 6



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